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Morphological and Molecular Study of *Trogoderma granarium* Everts, 1898in Iraq

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Abstract

Trogoderma granarium Everts 1898, commonly known as the khapra beetle, is a widespread pest species affecting stored grains. Samples of this pest were collected from different geographical regions across four governorates in Iraq, namely Mosul, Salahdin, Dhi Qar, and two regions within Baghdad (Latifiya and Jamila). The current study provides a detailed description of *T. granarium* about the morphological characteristics of the three body regions and their appendages that studied and supported by illustrations. Due to the morphological similarity between this species and many closely related species, accurate identification is challenging, particularly when the stored materials are contaminated with insect remains. Therefore, molecular diagnosis was confirmed using PCR-based assays such as conventional PCR and DNA sequencing, targeting mitochondrial (mtDNA) genes like COI (Cytochrome Oxidase I) and 16S (16S rRNA). Results showed that there are many genetic variations in the nitrogenous bases of the samples under study compared with the standard samples results which was taken from global data, with differences between the local samples themselves, which indicates the multiple genetic sources from which the insects came as a result of the import of grains and perhaps their poor storage.

Key words: *Trogoderma granarium*, Morphology, COI, 16Sr RNA

دراسة مظهرية و جزيئية لـ *Trogoderma granarium* Everts, 1898 في العراق

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الخلاصة

Trogoderma granarium Everts 1898، والمعروفة باسم خنفساء خابرا، هي نوع من الآفات واسعة الانتشار التي تؤثر على الحبوب المخزنة. تم جمع عينات من هذه الآفة من مناطق جغرافية مختلفة من أربع محافظات في العراق وهي الموصل وصلاح الدين وذي قار ومنطقتين داخل بغداد (اللطيفية وجميلة). تقدم الدراسة الحالية وصفا تفصيليا لـ *T. granarium* عن الخصائص الشكلية لمناطق الجسم الثلاثة وملحقاتها التي تمت دراستها ودعمها بالرسوم التوضيحية. ونظرًا للتشابه المورفولوجي بين هذا النوع

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والعديد من الأنواع ذات الصلة الوثيقة، فإن التحديد الدقيق يمثل تحديًا، خاصة عندما تكون المواد المخزنة ملوثة ببقايا الحشرات. لذلك، تم تأكيد التشخيص الجزيئي باستخدام فحوصات تعتمد على تفاعل البوليميراز المتسلسل مثل تسلسل تفاعل البوليميراز المتسلسل والحمض النووي التقليدي، واستهداف جينات الميتوكوندريا (mtDNA)مثلCOI (سيتوكروم اوكسايدI) و16S (16S rRNA). أظهرت النتائج وجود اختلافات وراثية كثيرة في القواعد النيتروجينية للعينات محل الدراسة مقارنة بنتائج العينات القياسية المأخوذة من البيانات العالمية، مع وجود اختلافات بين العينات المحلية نفسها، مما يدل على تعدد المصادر الوراثية التي جاءت منها الحشرات. نتيجة استيراد الحبوب وربما سوء تخزينها.

1. Introduction

The genus *Trogoderma* included the most well-known pest insects in the world and one of the most dangerous pests of stored products that related to global food security[1] . The introduction of invasive species that damage the environment and economy has risen due to escalating global trade connections. Globally there are more than 120 species belonged to this genus, but only four are known to be pests which infected stored grains, including *T. granarium, T.glabrum, T. inclusum, and T. variabile* [2]. In Iraq, five species of Trogoderma have been documented. These include *T. inclusum, T. granarium, T. variable, T. bactrianum, and T. irroraturn* [3]. These beetles tend to hide in cracks for a long period, and relatively tolerant to insecticides [4, 5] *T. granarium* is morphologically similar to several other *Trogoderma* species, therefore it is difficult to identify because of this reason many researchers use the molecular techniques. Regardless of life stage, species discrimination is possible because to molecular technology [6-11] .

Mitochondrial DNA (mtDNA) has gained widespread acceptance in animal research due to its relatively faster extraction process compared to nuclear DNA, and its ability to elucidate variations between closely related species more effectively[12] Mitochondrial cytochrome C oxidase I (COI) gene is one of the common DNA barcoding genes. COI known as the standard DNA barcoding for eukaryotic identification due to its relative conservatism. Other mitochondrial genes, such as the 16Sr RNA gene, can be used to identify species based on DNA sequence variation, Mitochondrial DNA has been used in many studies to find out the genetic diversity in populations taken from multiple regions [13-18]

The aim of this study is to provide a detailed description of external morphological characteristics and study genetic relationships between the studied specimens, which were collected from different geographical regions of Iraq. The phylogenetic tree revealed genetic diversity among the insect specimens, reflecting their multiple points of origin from different arrival sources.

2.Materials and Methods:

Samples collection:

More than 100 samples (male, female) were collected from four Governorates of Iraq Baghdad (Latifiya and Jamila), Mosul, Saladin and Dhi Qar for the period from Oct. 2020 to Dec. 2021, from infected grains such as wheat , barley , from local market, stores, silos, and preserved some of them on pin others in alcohol 70%. In addition the specimens compared with sample which stored at Iraq Natural History Museum, University of Baghdad to confirm this identification .The location and date of collection were provided, and then diagnosed using keys such as [19,20]

Molecular study:

By using Geneaid™ DNA Isolation Kit Tissue, (Taiwan) according to the protocol leaflet. the DNA was extracted from specimens, the quality of the DNA was measured using a Nano Drop spectrometer device from Biodrop UK, the purity of the DNA ranged between 1.8-2. Primer sets that have been chosen to amplification part of COI gene were the Universal primers LCO1490 and HCO2198, That use to investigate a wide range of invertebrate multicellular organisms[21,22] and 16Sr RNA primers LR-J-12961 and LR-N-13398 primers[22] Table (1). These primers were supplied by alpha DNA Company Canada. Each amplification reaction requires the addition of 1+1 µL of forward and reverse primers to the PCR mixture (Bioneer, Korea), which consists of 5 µL containing Taq polymerase, MgCl2, dNTPs (A, G, C, T), and loading dye. This is combined with 5 µL of DNA template and 13 µL of distilled water (D.D.W.), resulting in a final volume of 25 µL for the PCR reaction. Polymerase chain reaction products were verified by using 1.5% agarose gel electrophoresis in 1 X TBE buffer and stained with Safe- Red (Bioneer/ Korea) to visualize in ultraviolet light for 90 min at 70 volte Figure(1).

Table 1 : Sequence of primers utilized in this study.

Gene	Primer sequence	Primer name	Direction
COI	5′ -GGTCAACAAATCATAAAGATATTGG-3′	LCO1490	Forward
COI	5′-TAAACTTCAGGGTGACCA AAAAATCA-3′	HCO2198	Reverse
16Sr RNA	5′-TTTAATCCAACATCGAGG3′	LR-J-1296	Forward
16Sr RNA	5′-CGCCTGTTTAACAAAAACAT3	LR-N-13398	Reverse

Amplification of COI and 16s rRNA by using PCR

Detection of COI gene was conducted by using Pair of primers for the amplification amplifies a ~700 bp fragment of the mitochondrial COI gene and ~500bp fragment of the 16s rRNA gene, the temperature cycle conditions were performed as detailed in Tables (2) and (3).

Table 2: The optimum condition of COI gene detection

Steps	Stage	temperature	Time	No. of cycles
1	Initial Denaturation	94	4 min	1
2	Denaturation	94	30 sec	35
3	Annealing	58	30sec	
4	Extension	72	1min	

Table 3: The optimum condition of 16s gene detection.

Steps	Stage	temperature	Time	No. of cycles
1	Initial Denaturation	94	4 min	1
2	Denaturation	94	1 min	35
3	Annealing	55	1min	
4	Extension	72		
5	Extension Final	72	1min	4

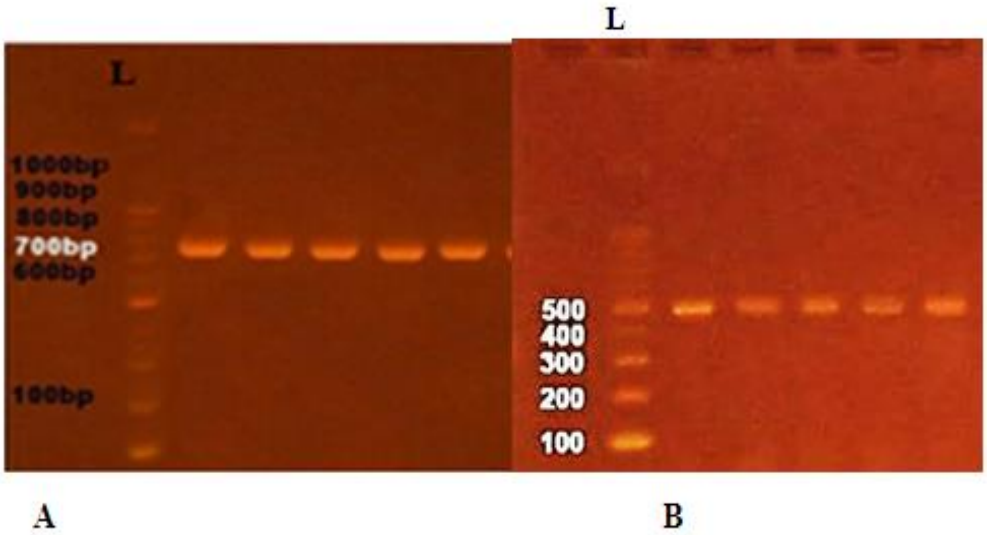

Figure 1: Gel electrophoresis for PCR product. The amplicons were run on agarose gel 1.5% in 70% Volts, 90 min and visualized with Trans illuminator A- COI gene B- 16s rRNA gene.

Sequencing of some PCR product sent to (Macrogen / Korea) with forward primer. The results were in the form of special files that were evaluated with Bioedit computer software. version7; 2013. The alignment and comparison of sequences generated from sequencing findings was also compared with data from the same organism genes that found in the gene bank at the National Center for Biotechnology (NCBI), which had previously been investigated in various nations across the world by using Basic Local Alignment Search Tool (BLAST). The results of sequencing had been showed (99 - 100 %) agreement with reference sequences. The phylogenetic tree of the species was constructed by uploading and analyzing the sequence data of the samples under study on the Phylogeny.fr web platform (http://www.phylogeny.fr/simple_phylogeny.cgi). The tree was generated using the online one-click workflow, which performed multiple sequence alignment, curation, phylogeny inference and tree rendering. The inferred tree was then compared to previously published trees of related species.

The nucleotide location value of variance is calculated using the maximum likelihood (ML), which ranges from 0 to 1. After comparing the alignment of the nitrogen bases among the samples, the highest probability of alterations in nucleotides revealed a genetic variation between the samples

3. Results and Discussion

3-1 Morphological study

Body

The body is oval-shaped and slightly convex on the dorsal side, with dense, dark brown to black hairs in males, as illustrated in Figure 2-a. Females are lighter than males Figure (2-b). Body length in male is 1.7-2.4 mm and width is 1.2-0.7 mm. In females, length is 2.1-3.3 mm; width is 1.6 -1.7mm.

Figure 2: *T. granarium* Everts, 1898 A-Male B- Female

Head:

The head is small in size, oval in shape, and brown to dark brown in coloration. Both the head and thorax are darker than the wings. The frons region contains a pair of simple eyes located medially between the two large compound eyes. Under magnification, the head surface can be seen to contain many small punctures as well as short, brown setae (hairs). The compound eyes are pale yellow with sinuous inner edges. The antenna (Figure 3) is brown to dark brown in color and is located inside a groove in the sides of the back of the anterior thoracic pronotum consisting of 11 segments ending in five clubs 1^{st}-2^{nd} antennomeres circular same sized 7^{th}-10^{th} antennomers nearly cup shaped and 10^{th} about 1.5 times as long as the 9^{th}. To distinguish between male and female, the last five antennomers in males are club-shaped, while in females, the last three antennomers are club-shaped. The antennomers contain few hairs.

Thorax:

The pronotum Figure (4) is dark brown to black, containing a small pit and covered with yellow hairs. The anterior margin is slightly curved while the posterior margin is pointed or resembles the letter V. The side edges are slightly curved, Scutellum is small triangular in shape, Figure (5). Legs yellow to brown Front legs Figure (6) short, procoxa oval, prochanter, triangular in shape, profemur cylindrical longer than tibia, protibia, tubular in shape, anterior front edge contains row of short spines, apical part contains 2 short spurs. The protarsus, or the foremost segment of the tarsus, comprises five articulated sections culminating in simple claws. The midlegs bear a resemblance to the forelegs, with the exception of the mesocoxa, which exhibits a circular, boat-like configuration. Similarly, the hindlegs mirror the forelegs in structure, save for the meta coxa, which adopts a boat-like shape and surpasses the femur in length. Middle legs are resembled to forelegs except, mesocoxa circular shaped, meso trochanter triangular. Hind leg Figure (7) is resembled to foreleg except, metacoxae, boat shaped, meta trochanter nearly oval shaped, meta tibia longer than the femur.

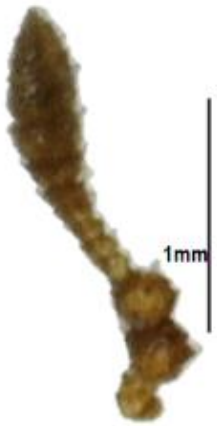

Figure 3: Antenna.

Figure 4: Prothorax**.**

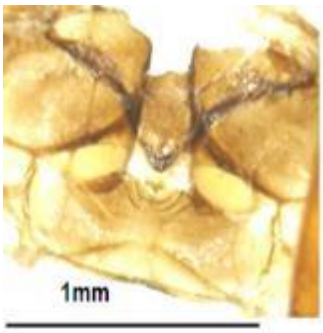

Figure 5: Scutellum.

Figure 6: Front leg. **Figure 7:** Hind leg.

Wings:

Wing Figure (8) Reddish-brown to dark brown in color, and the surface is covered with short seta and rounded pits of medium size.

Abdomen:

Abdomen Figure (9) Brown to dark brown the abdomen contains five sternal rings apparent The first sternal abdominal ring is 1.2 times longer than the second sternal abdominal ring. The second and fourth sternal abdominal rings are equal in length, the posterior edge of the fifth abdominal ring is round and covered with brown hairs.

Figure 8: Wing. **Figure 9:** Abdomen.

3-2 Molecular study:

Polymerase chain reaction primers of COI and 16s rRNA genes, and specific primers der were successfully amplified, gel electrophoresis was performed to show PCR amplification of the COI which yielded 700 bp and 16srRNA, which yielded a 500 bp product. These amplification techniques are employed for species identification and provide comparative data that contributes to developmental taxonomy studies and species-level family development research. Sequencing of these genes was performed in order to determine the genotype of *T. granarium* which were collected from four provinces of Iraq, Mosul, Baghdad (Latifiya and Jamila), Dhi Qar, and Saladin.

3-2-1 Study of DNA sequencing

The DNA sequencing is one of the most important methods that contributed to the rapid diagnosis of species[23] Molecular analysis helps in evaluating the heterogeneity between species in different geographic areas. The sequence of two genes examined forward primer only was performed, this was part of the sequence process requirements and the use of PCR technology in the context of the method of genetic analysis. The results of nucleotide alignment with the sequences in the gene bank showed that the identities ranged between 99-100%(Table 4).

3-2-2 Studying of COI Sequencing

The sequence of the COI gene part of the samples under study which is deposited in NCBI under accession number Table (4) was compared with the standard sequence of the same gene in Turkey, which is registered in NCBI under accession number NC_053875.1. The current study found similarities in the nucleotide sequences. When compared to results from amplifying the same gene fragment in samples from various geographical regions, it showed the insect originated from an introduction source in Turkey. The nucleotide sequence similarity across regions indicates the insect's initial point of entry and dispersal originated from Turkey. It was also found that there is genetic diversity in the sequences of the nitrogenous bases among the samples studied, that is, at the level of individual bases in the various governorates studied except for Baghdad Governorate, as it was observed that there are differences in the sequences of the nitrogenous bases of the samples under study with Standard samples, and this indicates the presence of genetic diversity for these species (Figure 10) through the presence of many variations in the gene for these species, as these differences represent the degree of diversity based on geographical location, which is agree with[24]

Table 4 : Accession number of local samples and the standard samples of the two genes, COI and 16s, identity.

Collection regions	Accession number in NCBI of studied sample of COI gene	Accession number in NCBI of studied sample of 16s gene	Accession number of standard references in NCBI	Identity
Baghdad/Latifiya	OM419213.1		NC_053875.1	100%
		OM388512	MZ571636.1.	100%
Mosul	OM407400.1		NC_053875.1	99.85%
		OM388538	MZ571636.1	99.75%
Dhi Qar	OM010359.1		NC_053875.1	99.68%
		OM388567	MZ571636.1	99.25%
Salahdin	OM407397		NC_053875.1	99%
		OM389131	MZ571636.1	99.25%
Baghdad/Jamila	M419213.1		NC_053875.1	100%
		OM389182	MZ571636.1	100%

3-2-3 Studying of 16s gene sequencing

The partial sequence of the 16S rRNA gene obtained from the studied samples, which is archived in the NCBI database under the accession numbers listed in Table 4, was compared against the reference sequence of the same gene from Australia, deposited in the NCBI under the accession number MZ571636.1. The sequence results for the 16s gene fragment showed There is a similarity in the sequence of nitrogenous bases between the species studied in different geographical regions. It was also noted that there are differences between the studied samples and the standard samples Figure (11).

The presence of genetic diversity in the form of single mutations in the gene fragment studied between the study samples and the standard reference sample confirmed that there is no complete match in the sequences of the nitrogen bases of this species, which is in agreement with some studies that confirm the existence of genetic differences due to geographical differences in sample collection[25]

3-3 The phylogenetic tree

The phylogenetic tree of the studied sample was drawn and compared with the standard sample in NCBI for two genes, COI, 16Sr RNA by using the website http://www.phylogeny.fr/simple_phylogeny.cgi.

The tree of genetic relations between studied samples showed that there were two sub-branches. The genetic variation between these two branches was 0.99. One of the branches included the samples of Mosul (M) and Saladin (S), and the second included Dhi Qar (DH), Baghdad [Latifiya (L) and Jamila (J)] and the standard sample (A). There was similarity between individuals of local samples (L), (J) and standard sample (A) the ratio was 0.75, Figure (12). Also, it was found that there is a genetic variation between the samples for 16Sr RNA gene when drawing phylogenetic tree there are two branches one of them have the ratio of 0.92 for local sample S and DH and the second also have two branches one for local sample M and the percentage of genetic variation was 0.9 and others for There were similarity between individual of local sample (L), (J) and standard sample (A) and the ratio was 0.84. Figure (13). Environmental factors, such as biotic or abiotic, were the main motivators for the formation of subunits within the population also resistant to pesticides[26]

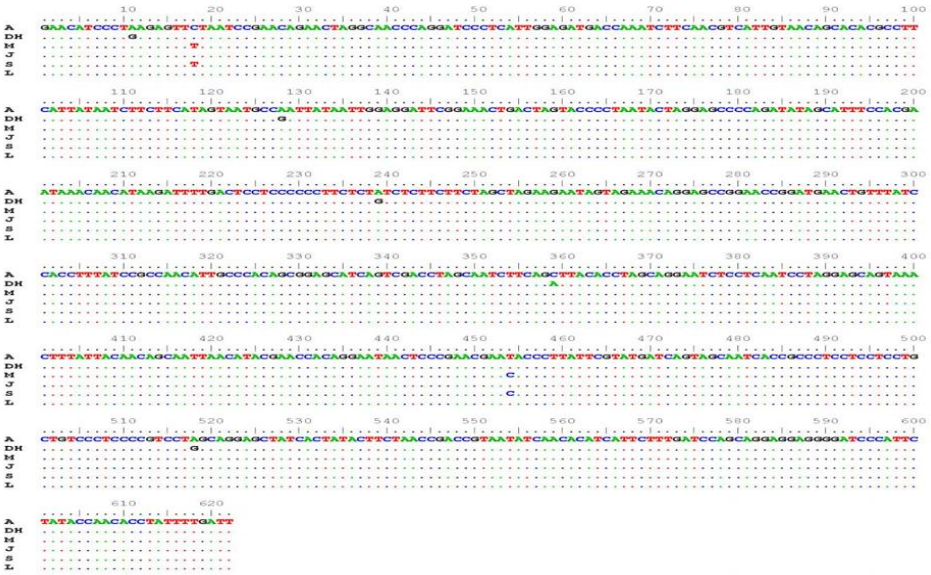

Figure 10 : The alignment of Cytochrome Oxidase I gene in local samples Dhi Qar (DH), Mosul (M) Jamila (J) and Saladin (S), Latifiya (L) with the standard sequence (A).

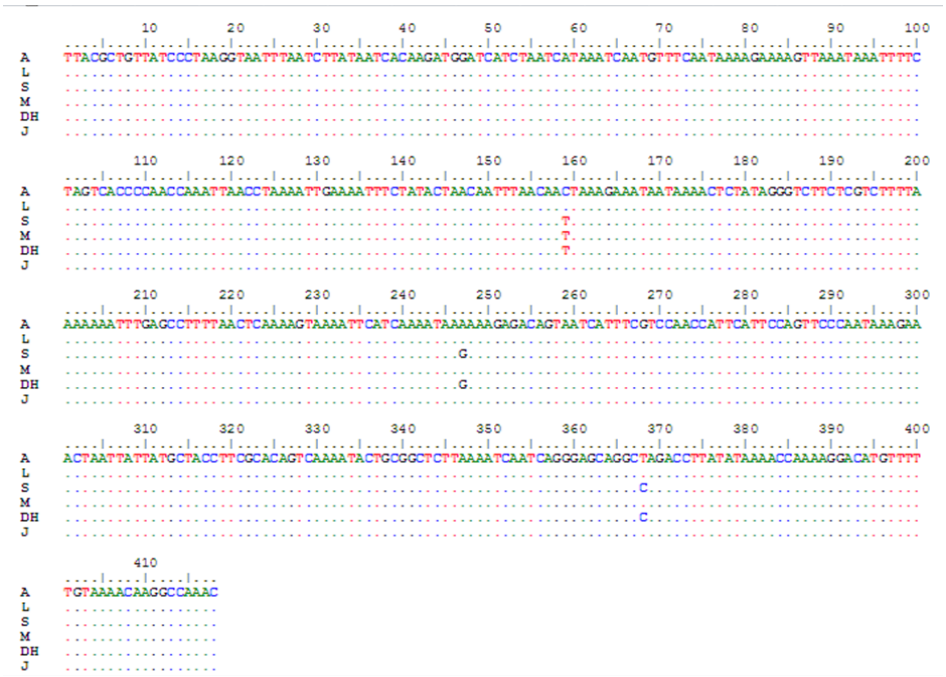

Figure 11 : The alignment of 16s rRNA gene in local samples Dhi Qar (DH), Mosul (M) Jamila (J) and Saladin (S), Latifiya (L) with the standard sample (A).

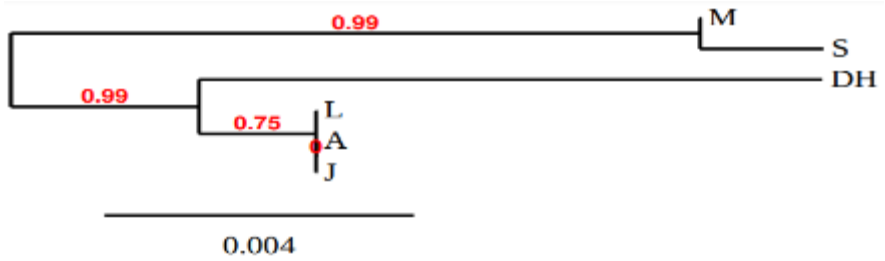

Figure 12 : Phylogenetic tree of Cytochrome Oxidase I gene sequences in *T. granarium* Dhi Qar (DH), Mosul (M) Jamila (J) and Saladin (S), Latifiya (L) with the standard sample (A).

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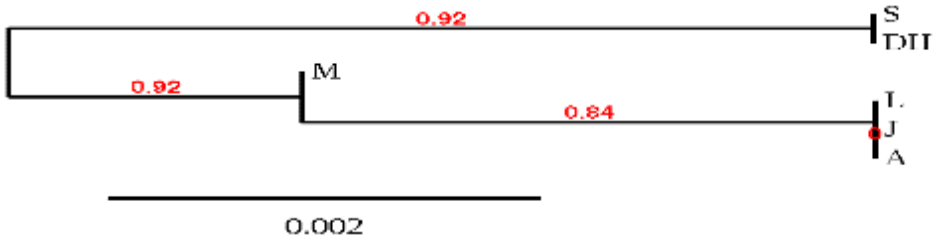

Figure 13 : Phylogenetic tree of 16s gene sequences in *T. granarium* Dhi Qar (DH), Mosul (M) Jamila (J) and Saladin (S), Latifiya (L) with the standard sample (A).

Conclusions

Based on the currently available data from this study, it has been shown that both mtDNA genes COI and 16s rRNA successful amplified in PCR. Additionally, the presence of multiple mutations within the gene further substantiates the genetic variability observed among these samples. These differences in the genetic makeup are indicative of the diversity that exists based on geographical origins. As it may be considered environmental factors (biotic or

abiotic) and resistance to pesticides are the main motivators for the diversity and the formation of subunits within the population.

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